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Remarks:
The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) **Method for obtaining modified immunoglobulins with reduced immunogenicity of murine antibody variable domains, compositions containing them**

(57) Modified chimaeric antibodies, and antibody heavy and light chains, which comprise variable domains derived from a first mammalian species, usually mouse, and constant domains from a second mammalian species, usually human. Modification concerns the variable domains, in particular the framework regions of the variable domains. The modifications are made only in T-cell antigenic structures present in framework regions, and do not cover canonical structures or Vernier zone. The modifications adapt the amino acid sequences concerned to those occurring in corresponding antibodies derived from said second mammalian species. Thus, the modified chimaeric antibodies retain the original antigen recognition and binding properties but become less immunogenic to said second mammalian species, which improves their therapeutical utility with said second mammalian species. Recombinant DNA technology may be used to construct and produce the modified chimaeric antibodies.

FIGURE 1: DEDUCED AMINO ACID SEQUENCES

A VK OF MURINE R3 ANTIBODY

D V L M T Q I P L S L P V S L G D Q A S I S C **R S S Q**
N I N I V H S N G N T Y L D W Y L Q K P G Q S P N L L
I Y **K V S N R F S** G V P D R F R G S G S G T D F T L K
I S R V E A E D L G V Y Y C **F Q Y S H V P W T** F G G G
T K L E I K R A

B VH OF MURINE R3 ANTIBODY

Q V Q L Q Q P G A E L V K P G A S V K L S C K A S G Y
T F T **N Y Y I Y** W V K Q R P G Q G L E W I G **G I N P T**
S G G S N F N E K F K T K A T L T V D E S S T T A Y M
Q L S S L T S E D S A V Y Y C T R **Q G L W F D S D G R**
G F D Y W G Q G T T L T V S S

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A further procedure for the humanization of an antibody has been suggested by Padlan (Padlan, European Patent Application, Publication No. 0 519 596 A1; Padlan, Molecular Immunology 28: 489-498 (1991)). It is based on the fact that the antigenicity of a protein is dependent on the nature of its surface, and a number of the solvent-accessible residues in the rodent variable region are substituted by residues from a human antibody. The locations of these residues are identified from an inspection of the high resolution X-ray structures of the human antibody KOL and the murine antibody J539. The choice of the human surface residues is arrived at by identifying the most homologous antibody sub-group.

The nature of the protein surface is important for its recognition and internalization by antigen-processing cells, specifically by antigen-specific B-cells. In addition, the recognition of specific linear sequences by T-cells is also an important element in the immunogenicity of proteins.

Several groups have developed automated-computerized methods for the identification of sequence features and structural determinants that play a role in the MHC restriction of helper T-cell antigenic peptides (Bersofsky et al, J. Immunol. 138: 2213-2229 (1987), Elliott et al, J. Immunol. 138: 2949-2952 (1987), Reyes et al, J. Biol. Chem. 264: 12854-12858 (1989)). Using these algorithms, it has been possible to identify predicted T cell-presented peptides.

Analysis of antibodies of known atomic structure has elucidated relationships between the sequence and three-dimensional structure of antibody combining sites (Chothia et al, J. Biol. Chem. 196: 901-917 (1987)). These relationships imply that, except for the third region in the VH domains, binding site loops have one of a small number of main-chain conformations: "Canonical structures". The canonical structure formed in a particular loop is determined by its size and the presence of certain residues at key sites in both the loop and in framework regions.

An additional subset of framework residues has been defined as "Vernier" zone, which may adjust CDR structure and fine-tune the fit to antigen (Foot et al, J. Mol. Biol. 224: 487-499 (1992)). Substitutions of these residues have been shown to be important to restoring the affinity in CDR grafted antibodies, so the Vernier zone has an obvious consequence for the design of humanized antibodies.

SUMMARY OF THE INVENTION

It is, accordingly, an objective of the present invention to provide a means of converting a monoclonal antibody of one mammalian species to a monoclonal antibody of another species. Another object is to predict potential T-epitopes within the sequence of variable regions. Another object is to identify the amino acid residues responsible for species specificity or immunogenicity within the sequence of the monoclonal antibody responsible of the T-immunogenicity. Another object is to judiciously replace the amino acid residues within the T-epitope sequences of one species with those of a second species so that the antibodies of the first species will not be immunogenic in the second species. A further object is to make replacements only in the framework regions of the heavy and light chains and not in the complementarity determining regions; also the amino acids belonging to the Vernier zone and those involved in the canonical structures cannot be replaced. Another object of the invention is to provide novel DNA sequences incorporating the replacement amino acid residues. Another object is to provide a vector containing the DNA sequences for the altered antibody. Another object is to provide a eukaryotic or procaryotic host transformed with a vector containing the DNA sequence for the modified antibody.

A unique method is disclosed for identifying and replacing amino acid residues within T-cell antigenic sequences which converts immunoglobulin antigenicity of a first mammalian species to that of a second mammalian species. The method will simultaneously change immunogenicity and strictly preserve ligand binding properties. A judicious replacement of those amino acid residues within T-cell antigenic sequences of the variable regions, which are not involved in the three-dimensional structure, has no effect on the ligand binding properties but greatly alters immunogenicity.

BRIEF DESCRIPTION OF THE DRAWINGS.

FIGURE 1: Deduced amino acid sequence of (a) VK and (b) VH of murine R3 antibody. CDRs are underlined.
FIGURES 2 and 3: Analysis for the modification of the variable regions of heavy and light chains of antibody IOR-R3.

A: sequence of the variable region of the murine IOR-R3 monoclonal antibody.
B: sequence of the variable region of the most homologous human immunoglobulin.
C: sequence of the modified variable region of IOR-R3.

shading: predicted T-cell antigenic sequences.
underlined amino acid residues: amino acids involved in tertiary structure.
bold font: complementarity determining regions.
amino acid residues in boxes: proposed replacements.
The description is the same for both, heavy and light chains.

(3) Analysis for immunogenicity reduction

Those residues in the mouse framework which differ from its human counterpart are replaced by the residues present in the human counterpart. This switching (replacement) occurs only with those residues which are in the T-antigenic sequences.

Finally, replacement of those residues responsible for the canonical structures or those involved in the Vernier zone could have a significant effect on the tertiary structure. Hence, they cannot be included in the replacement. Additional information about the influence of the proposed replacements on tertiary structure or the binding site could be obtained from a molecular model of the variable regions.

The molecular model can be built on a Silicon Graphics Iris 4D workstation running UNIX and using the molecular modeling package "QUANTA" (Polygen Corp.).

(4) Method for constructing and expressing the altered antibody

The following procedures are used to prepare recombinant DNA sequences which incorporate the CDRs of a first mammalian species, usually animal, e.g. murine mAb, both light and heavy chains, into a second mammalian species, preferably human, appearing frameworks that can be used to transfect mammalian cells for the expression of recombinant antibody less immunogenic and with the antigen specificity of the animal monoclonal antibody.

The present invention further comprises a method for constructing and expressing the modified antibody comprising:

a.-) mutagenesis and assembly of variable region domains including CDRs and FRs regions. The PCR-mutagen sis method (Kamman et al, Nucleic Acids Res. 17: 5404-5409 (1989)) is preferably used to introduce the changes at different positions.
b.-) preparation of an expression vector including one variable region and the corresponding human constant region which upon transfection into cells results in the secretion of protein sufficient for affinity and specificity determinations.
c.-) co-transfection of heavy and light chain expression vectors in appropriate cell lines.

After about 2 weeks, the cell supernatants are analyzed by ELISA for human IgG production. The samples are then analysed by any method for human IgG capable of binding to specific antigens.

The present invention provides a method for incorporating CDRs from animal monoclonal antibodies into frameworks which appear to be human immunoglobulin in nature so that the resulting recombinant antibody will be either weakly immunogenic or non-immunogenic when administered to humans. Preferably the recombinant immunoglobulins will be recognized as self proteins when administered for therapeutic purpose. This method will render the recombinant antibodies useful as therapeutic agents because they will be either weakly immunogenic or non-immunogenic when administered to humans.

The invention is further contemplated to include the recombinant conversion of any animal monoclonal antibody into a recombinant human-appearing monoclonal antibody by providing that with a suitable framework region.

The invention is intended to include the conversion of any animal immunoglobulin to a human-appearing immunoglobulin. It is further intended that human-appearing immunoglobulin can contain either Kappa or Lambda light chains or be one of any of the following heavy chain isotypes (alpha, delta, epsilon, gamma and mu).

The following examples intend to ilustrate the invention but not to limit the scope of the invention.

EXAMPLE 1: Murine Variable region of R3 monoclonal antibody DNA sequencing

Cytoplasmic RNA was extracted from about 10^6 R3 (anti Epidermal growth Factor receptor) hybridoma cells as described by Faloro et al (Faloro, J. et al, Methods in Enzymology 65: 718-749, 1989).

The cDNA synthesis reaction consisted of 5 ug RNA, 50 mM Tris-HCl, pH 7.5, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 25 pmol of CG2AFOR primer (5' GGAAGCTTAGACCGATGGGGCCTGTTGTTTTG 3') for the heavy chain variable region or CK2FOR primer (5' GGAAGCTTGAAGATGGATACAGTTGGTGCAGC 3') for the light chain variable region, 250 uM each of dATP, dTTP, dCTP, dGTP, 15 U ribonuclease inhibitor (RNA guard, Pharmacia) in a total volume of 50 ul. Samples were heated at 70°C for 10 min and slowly cooled to 37°C over a period of 30 min. Then, 100 units MMLV reverse transcriptase (BRL) were added and the incubation at 37°C continued for 1 hour.

The VH and VK cDNAs were amplified using the PCR as described by Orlandi et al (Orlandi, R. et al, Proc. Natl. Acad. Sci. USA 86: 3833-3837, 1989). For PCR amplification of VH, DNA/primer mixtures consisted of 5 ul cDNA, 25 pmoles of CG2AFOR primer (5' GGAAGCTTAGACCGATGGGGCCTGTTGTTTTG 3') and VH1BACK primer (5' AGGT(G/C)(A/C)A(A/G)CTGCAG(G/C)AGTC(A/T)GG 3').

For PCR amplification of VK, DNA/primers mixtures consisted of 5 ul cDNA and 25 pmol s of CK2FOR primer (5' GGAAGCTTGAAGATGGATACAGTTGGTGCAGC 3') and VK10BACK primer (5' TTGAATTCCAGTGATGTTTTGATGACCCA 3'). To these mixtures were added 2.5 mM each of dATP, dCTP, dTTP, and dGTP, 5 ul constituents of 10X

The T-cell antigenic sequence in the FR2 contains two PRO which is a very rare amino acid residue in most of the helical antigenic sites, so we propose that it is not a real T-cell epitope.

In the position 108 at the FR4 appears THR which is present in the same position in some human immunoglobulins, only residue 109 (LEU) is very rare in human, except for this point difference most of the predicted T-cell epitope is human, on this basis it does not need to be modified.

In Figure 3 the analysis for the light chain of murine R3 is shown.

In the sequence only one amphipatic helix was predicted, between residue 52-63 corresponding to CDR2 and FR3, and in this region only one point difference exists between murine and human sequences, at position 63. No replacement is proposed, because this murine light chain should be non-immunogenic in human (see molecular modelling).

EXAMPLE 4: Molecular Modelling of mAb R3 VK and VH

A model of the variable regions of mouse mAb R3 was built using the molecular modeling program QUANTA/CHARm 4.0 (Molecular Simulations Inc., 1994), running on a 150 MHz Silicon Graphics Indigo Extreme workstation. The VK and VH frameworks were built separately from Fab 26-10 (Jeffrey, P.D et al, Proc. Natl. Acad. Sci. USA 90, 10310, 1993) and Fab 36-71 (Strong, R.K. et al, Biochemistry 30, 3739, 1993), respectively. Fab 26-10 and mAb R3 have 92% homology in the VK frameworks and 88% homology in the whole VK region. The VH frameworks of Fab 36-71 and mAb R3 have 85% homology.

Coordinates were taken from the Brookhaven Protein Data Bank (entries 1IGI and 6FAB). The frameworks of Fab 36-71 were fitted to the frameworks of Fab 26-10, matching only those residues that have been found to be often involved in the interface between the light and heavy variable regions (Chotia, C. et al, J. Mol. Biol. 186, 651, 1985). The VH domain of Fab 26-10 and the VK domain of Fab 36-71 were then deleted leaving the needed hybrid. Side-chain replacements were performed following the maximum overlap procedure (Snow, M.E. et al, Proteins 1, 267, 1986) and comparing, where possible, with other crystal structures.

The hypervariable regions of the R3-Variable Light (VL) domain (L1, L2 and L3) were built retaining the same main-chain conformations as in Fab 26-10, since the corresponding CDRs in both antibodies are highly homologous and belong to the same canonical structural groups (Chotia, C. et al, Nature 342, 877, 1989). In the VH domain of mAb R3, CDR H1 belongs to canonical structural group 1, as in Fab 36-71, so the main-chain torsion angles of the parent molecule were kept. CDR H2 corresponds to canonical structural group 2 and the main-chain conformation for this loop was taken from the Fv fragment 4D5 (entry 1FVC), which was selected among other highly resolved structures because of the good matching of its H2 loop base with the framework of Fab 36-71. For all the above mentioned loops comparisons with other CDRs from the Data Bank were made to orient the side chains.

To model CDR H3, which in mAb R3 was 14 amino acids long, a high temperature molecular dynamics was used for conformational sampling (Bruccoleri, R.E. et al, Biopolymers 29, 1847, 1990). First, the whole structure without CDR H3 was subjected to an energy minimization keeping residues H-94 and H-103 fixed and using harmonic constraints of 10 Kcal/(mole atom A^2) for main chain atoms. Then a loop was constructed with an arbitrary conformation starting from the two previously fixed amino acids. Those residues close to the framework were placed taking into consideration other crystal structures and the top part of the loop was built with an extended conformation avoiding strong steric interactions with the rest of the molecule. For the next modeling steps only CDR H3 and the neighbouring side chains within a distance of $5A^0$ were permitted to move. An energy minimization was first carried out and then a molecular dynamics at 800 K was run for 150 picoseconds. The time step for the run was set to 0.001 picosecond and coordinates were saved every 100 steps. The 120 lowest energy conformations from the dynamics run were extracted and subjected to an energy minimization in which all atoms in the structure were allowed to move. Several low-energy conformations were obtained and the one with the lowest energy was used in the subsequent analyses. Differences between murine and humanized variants of R3 antibody were individually modeled to investigate their possible influence on CDR conformation.

Amino acid replacements in positions 11, 12 (FR1) and 83 (FR3) in the heavy chain variable region are quite enough distant from the CDRs-FRs boundaries and should not have any influence on binding affinity. SER 75 residue is pointing to outside, thus the replacement by THR seems not to be important for binding capacity. By contrary THR 76 is accessible from the top of the molecule and could be involved in the interaction with the antigen. But the substitution of THR 76 by SER is a conservative change, leading to no major variations in binding affinity probably.

The replacement of ALA 78 by VAL should not require steric rearrangements. However VAL 78 could "push" forward ILE 34 (H!). In general, the proposed point mutations should not affect binding affinity according to the computer-aided molecular modelling study (Figure 4).

The same analysis was done in the light chain variable region of IOR-R3, molecular modelling indicates it is not necessary to make any changes in this region.

M), confirming that the correct mouse variable regions had been cloned and the new antibody isotype did not affect binding. Even more, the changes in the mutant antibody did not affect binding to the antigen.

EXAMPLE 9: Immunization of Cercopithecus aethiops monkeys with the murine, chimaeric and VH mutant antibodies

Three treatment groups with two Cercopithecus aethiops monkeys in each group were immunized with murine R3 mAb, chimaeric R3 antibody and mutant VH R3 antibody, respectively. All the groups were immunized subcutaneously on days 0, 14, 28 and 42, with 2 mg of antibody adsorbed into 5 mg of aluminum hydroxide.

Blood was collected prior to the first immunization and one week later of each immunization, from all the groups, and the serum was obtained from each sample, and kept at -20°C. The titer of antibodies against the murine R3 mAb was determined by an ELISA technique.

Costar plates (Inc, high binding) were coated with murine R3 monoclonal antibody at a concentration of 10 ug/ml in bicarbonate buffer (pH 9.6) and incubated overnight. Thereafter, the plates were washed with PBST, were blocked with the same buffer containing 1% BSA during one hour at room temperature.

The washing step was repeated and 50 ul/well of the different serum dilutions were added. After incubating for 2 hours at 37°C, the plates were washed again and incubated 1 hour at 37°C with alkaline phosphated conjugated goat anti-human total or anti-human IgG Fc region specific antiserum (Sigma, Inc). After washing with PBST the wells were incubated with 50 ul of substrate buffer (1 mg/ml of p-nitrophenylphosphate diluted in diethanolamine buffer (pH 9.8)). Absorbance at 405 nm in an ELISA reader (Organon Teknika, Inc).

A high IgG response to murine R3 antibody was obtained when this antibody was used as immunogen. A lower but still measurable IgG response (1 / 10 000) to the murine R3 antibody was obtained when monkeys were immunized with the chimaeric antibody, contrary to the results obtained with the mutant Vh version (Figure 6). With the mutant VH R3 antibody no response was measurable after two immunizations, and a small response (1 / 10 000) was measured after 4 immunizations.

EXAMPLE 10: Modification of the variable domain sequences of IOR-T1 murine monoclonal antibody to humanize the predicted T-cell antigenic sequences

The variable region sequences of heavy and light chains of IOR-T1 were analyzed for T-cell antigenic sequences. In the variable domain of the heavy chain 3 segments were predicted, they are:

1. FR1 between amino acids 2-21.
2. FR1, CDR1, FR2 between amino acids 29-43.
3. FR4, CDR3 between amino acids 97-111.

FIGURE 7 shows a comparison with the most homologous human sequence and the replacement proposed, which are 5 at the FR1, 2 at the FR2 and 2 at the FR4.

The same procedure with the light chain (Figure 8) rendered the following T-cell antigenic segments:

1. FR3 between amino acids 60-65.
2. FR3, CDR3 between amino acids 79-90.
3. CDR3 between aminoacids 93-95A.

After the analysis we proposed 5 replacement in FR3 at positions: 60, 63, 83, 85 and 87.

EXAMPLE 11: Modification of the variable domain sequences of IOR-CEA1 murine monoclonal antibody to humanize the predicted T-cell antigenic sequences

The variable region sequences of heavy and light chains of IOR-CEA1 were analyzed for T-cell antigenic sequences. In the variable domain of the heavy chain two segments were predicted, they are:

1. FR1 between amino acids 1-16.
2. CDR3 and FR4 between residues 96-110.

FIGURE 9 shows a comparison with the most homologous human sequence and the replacements proposed, which are 7 at the FR1 and 2 at the FR4.

The same analysis with the light chain (Figure 10) rendered the following T-cell antigenic segments:

1. FR1 between amino acids 1-14.

predicted T-cell epitopes by few point mutations.

SEQUENCE LISTING

(1, GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Method for obtaining modified immunoglobulins with reduced immunogenicity of murine antibody variable domains, compositions containing them.

(iii) NUMBER OF SEQUENCES: 13

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: EP 95201752.3

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGAAGCTTAG ACCGATGGGG CCTGTTGTTT TG 32

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

TGAGGAGACG GTGACCGTGG TCCCTTGGCC CCAG 34

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GACATTCAGC TGACCCA 17

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTTAGATCTC CAGTTTGGTG CT 22

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAAGCCCCAG GCTTCTTCAC TTCAGCCCCA GGCTG 35

(2) INFORMATION FOR SEQ ID NO: 9:

(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GCAGAGTCCT CAGATCTCAG GCTGCTGAGT TGCATGTAGA CTGTGCTGGT GGATTCGTCT 60

ACCGT 65

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ACGGTAGACG AATCCACCAG CACAGTCTAC ATGCAACTCA GCAGCCTGAG ATCTGAGGAC 60

TCTGC 65

Claims

1. A method for identifying differences in mammalian species specific amino acid residues within T-cell antigenic sequences in an immunoglobulin, comprising

   a. comparing the framework amino acids of a variable domain of a first mammalian species with the variable domains of a second mammalian species;
   b. determining the subgroups of the second mammalian species to which the first mammalian species most closely corresponds;
   c. determining the second mammalian species sequence which is most similar to the first mammalian species sequence;
   d. identifying amino acid residues of the first mammalian species which differ from the amino acid residues of the second mammalian species, with said amino acids being within T-cell antigenic sequences in the variable region of the immunoglobulins;
   e. identifying only those amino acid residues which are not within a complementarity region or are not directly involved with canonical structures or Vernier zone.

2. The method of claim 1 wherein the first mammalian species is mouse.

3. The method of claim 1 wherein the second mammalian species is human.

FR3: ASP by ALA at position 60, THR by SER at position 63, LEU by PHE at position 83, GLU by VAL at position 85, PHE by TYR at position 87.

19. A pharmaceutical composition comprising a modified chimaeric monoclonal antibody according to claim 17 or claim 18

20. A DNA sequence encoding modified chimaeric IOR-CEA1 antibody obtained by methods of claims 1, 4 and 7.

21. A modified chimaeric IOR-CEA1 antibody exhibiting the antigenicity of human antibody obtained by methods of claims 1, 4 and 7.

22. A modified chimaeric IOR-CEA1 antibody according to claim 21 with the following point mutations in the framework regions of both chains:
Heavy chain:
FR1: PRO by VAL at position 2, LYS by GLN at position 3, LEU by VAL at position 5, GLU by GLN at position 6, GLY by ALA at position 9, ASP by GLU at position 10, GLU by GLY at position 15;
FR4: THR by LEU at position 108, LEU by VAL at position 109; Light chain:
FR1: LYS by SER at position 9, PHE by THR at position 10, SER by LEU at position 11, THR by ALA at position 13;
FR3: VAL by ILE at position 58, ASP by SER at position 60, THR by SER at position 63, ASP by GLU at position 70, ILE by VAL at position 75, SER by ILE at position 76, VAL by LEU at position 78, GLN by ASP at position 81, LEU by PHE at position 83, GLU by THR at position 85, PHE by TYR at position 87;
FR4: ALA by GLN at position 100.

23. A pharmaceutical composition comprising a modified chimaeric monoclonal antibody according to claim 21 or claim 22.

24. The therapeutic use of modified chimaeric monoclonal antibodies according to any one of claims 13, 14, 17, 18, 21 and 22.

25. Use of modified chimaeric antibodies according to any one of claims 13, 14, 17, 18, 21 and 22 for the manufacture of a drug directed to tumors.

26. A modified chimaeric antibody comprising heavy and light chain variable domains derived from a first mammalian species and heavy and light chain constant domains derived from a second mammalian species, wherein the heavy chain variable domain or the light chain variable domain, or both, is modified within T-cell antigenic sequences to adapt the amino acid sequence to the amino acid sequence in the corresponding regions of an antibody derived from said second mammalian species, with the exclusion of modifications in the complementarity determining regions, and modifications in the canonical structures or Vernier zone within the framework regions.

27. A modified chimaeric antibody heavy chain comprising heavy chain variable domains derived from a first mammalian species and heavy chain constant domains derived from a second mammalian species, wherein the heavy chain variable domain is modified within T-cell antigenic sequences to adapt the amino acid sequence to the amino acid sequence in the corresponding heavy chain regions of an antibody derived from said second mammalian species, with the exclusion of modifications in the complementarity determining regions, and modifications in the canonical structures or Vernier zone within the framework regions.

28. A modified chimaeric antibody light chain comprising light chain variable domains derived from a first mammalian species and light chain constant domains derived from a second mammalian species, wherein the light chain variable domain is modified within T-cell antigenic sequences to adapt the amino acid sequence to the amino acid sequence in the corresponding light chain regions of an antibody derived from said second mammalian species, with the exclusion of modifications in the complementarity determining regions, and modifications in the canonical structures or Vernier zone within the framework regions.

FIGURE 2: VARIABLE REGION OF THE HEAVY CHAIN OF IOR-R3.

	1	2	3	4	5	6	7	8	9	10	11	12
A	GLN	VAL	GLN	LEU	GLN	GLN	PRO	GLY	ALA	GLU	LEU	VAL
B	GLN	VAL	GLN	LEU	VAL	GLN	SER	GLY	ALA	GLU	VAL	LYS
C	GLN	VAL	GLN	LEU	GLN	GLN	PRO	GLY	ALA	GLU	VAL	LYS
	13	14	15	16	17	18	19	20	21	22	23	24
A	LYS	PRO	GLY	ALA	SER	VAL	LYS	LEU	SER	CYS	LYS	ALA
B	LYS	PRO	GLY	ALA	SER	VAL	LYS	VAL	SER	CYS	LYS	ALA
C	LYS	PRO	GLY	ALA	SER	VAL	LYS	LEU	SER	CYS	LYS	ALA
	25	26	27	28	29	30	31	32	33	34	35	36
A	SER	GLY	TYR	THR	PHE	THR	ASN	TYR	TYR	ILE	TYR	TRP
B	SER	GLY	TYR	THR	PHE	ASN						TRP
C	SER	GLY	TYR	THR	PHE	THR	ASN	TYR	TYR	ILE	TYR	TRP
	37	38	39	40	41	42	43	44	45	46	47	48
A	VAL	LYS	GLN	ARG	PRO	GLY	GLN	GLY	LEU	GLU	TRP	ILE
B	VAL	ARG	GLN	ALA	PRO	GLY	GLN	GLY	LEU	GLU	TRP	MET
C	VAL	LYS	GLN	ARG	PRO	GLY	GLN	GLY	LEU	GLU	TRP	ILE
	49	50	51	52	52A	53	54	55	56	57	58	59
A	GLY	GLY	ILE	ASN	PRO	THR	SER	GLY	GLY	SER	ASN	PHE
B	GLY											
C	GLY	GLY	ILE	ASN	PRO	THR	SER	GLY	GLY	SER	ASN	PHE
	60	61	62	63	64	65	66	67	68	69	70	71
A	ASN	GLU	LYS	PHE	LYS	THR	LYS	ALA	THR	LEU	THR	VAL
B							ARG	VAL	THR	MET	THR	ARG
C	ASN	GLU	LYS	PHE	LYS	THR	LYS	ALA	THR	LEU	THR	VAL
	72	73	74	75	76	77	78	79	80	81	82	82A
A	ASP	GLU	SER	SER	THR	THR	ALA	TYR	MET	GLN	LEU	SER
B	ASP	THR	SER	THR	SER	THR	VAL	TYR	MET	GLU	LEU	SER
C	ASP	GLU	SER	THR	SER	THR	VAL	TYR	MET	GLN	LEU	SER
	82B	82C	83	84	85	86	87	88	89	90	91	92
A	SER	LEU	THR	SER	GLU	ASP	SER	ALA	VAL	TYR	TYR	CYS
B	SER	LEU	ARG	SER	GLU	ASP	THR	ALA	VAL	TYR	TYR	CYS
C	SER	LEU	ARG	SER	GLU	ASP	SER	ALA	VAL	TYR	TYR	CYS
	93	94	95	96	97	98	99	100	100A	100B	100C	100D
A	THR	ARG	GLN	GLY	LEU	TRP	PHE	ASP	SER	ASP	GLY	ARG
B	ALA	ARG										
C	THR	ARG	GLN	GLY	LEU	TRP	PHE	ASP	SER	ASP	GLY	ARG
	100E	100F	101	102	103	104	105	106	107	108	109	110
A	GLY	PHE	ASP	PHE	TRP	GLY	GLN	GLY	THR	THR	LEU	THR
B					TRP	GLY	GLN	GLY	THR	LEU	VAL	THR
C	GLY	PHE	ASP	PHE	TRP	GLY	GLN	GLY	THR	THR	LEU	THR
	111	112	113									
A	VAL	SER	SER									
B	VAL	SER	SER									
C	VAL	SER	SER									

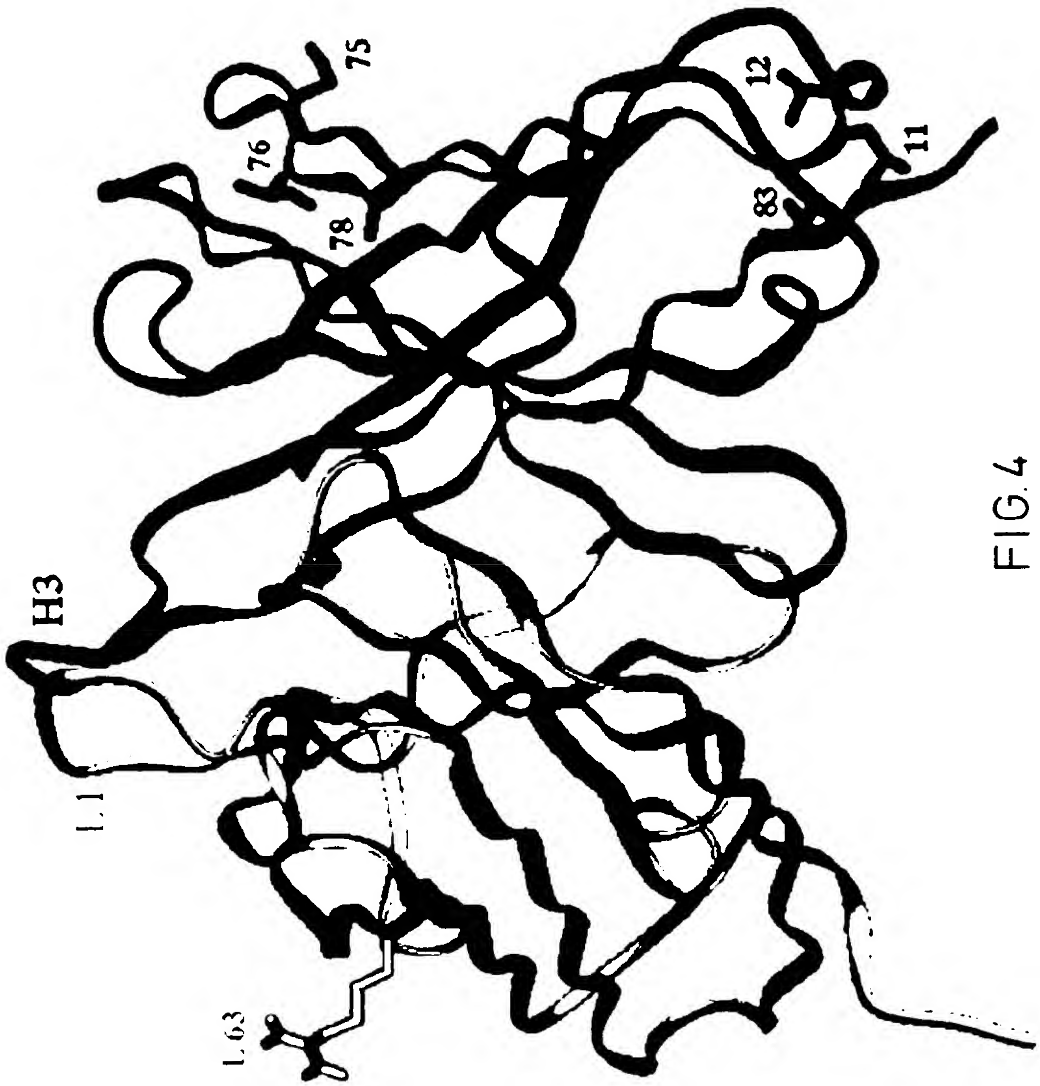
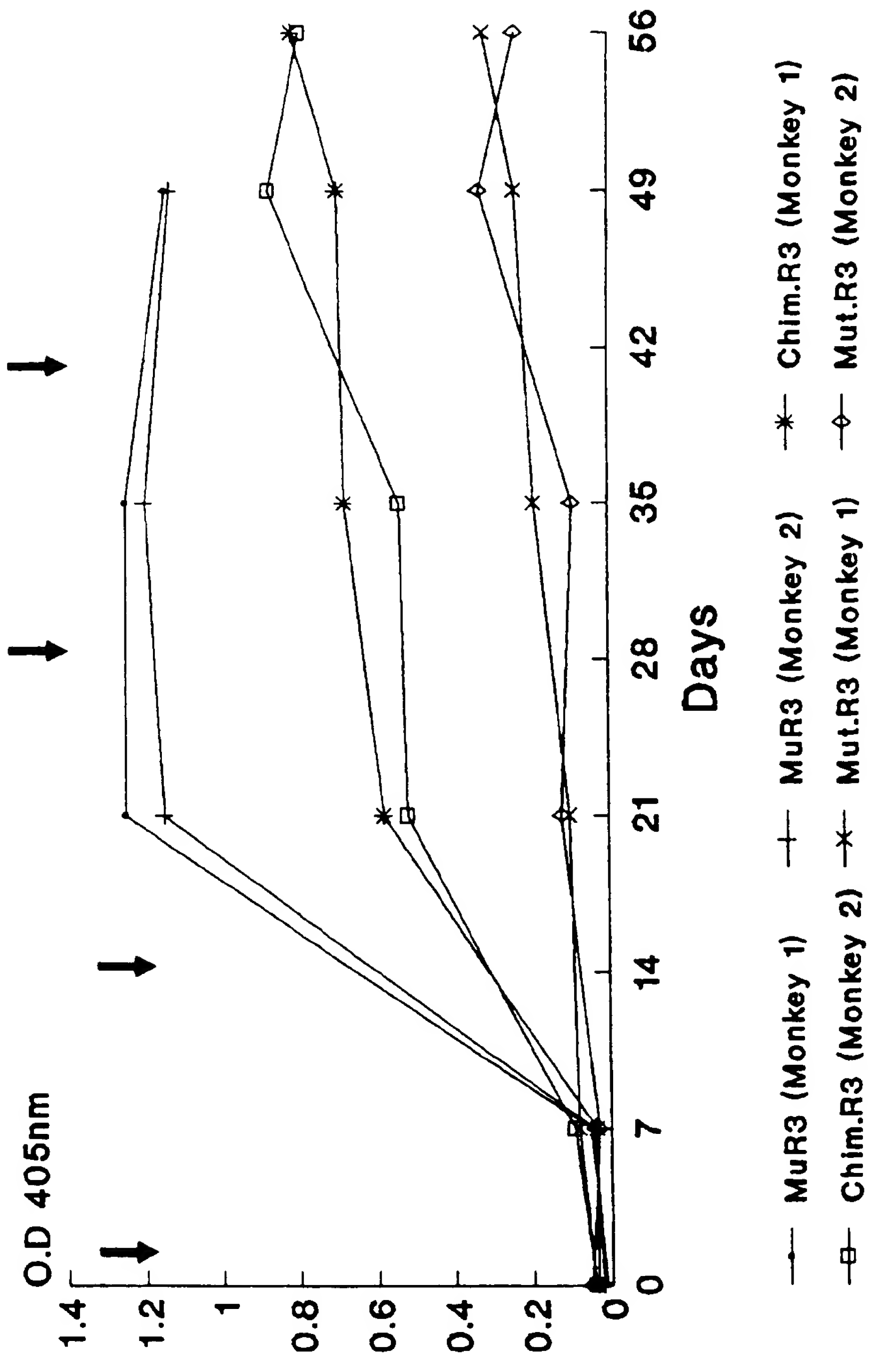


FIG. 4

Figure 6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1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
7
14
21
28
35
42
49
56
Days
MuR3 (Monkey 1)
MuR3 (Monkey 2)
Chim.R3 (Monkey 1)
Chim.R3 (Monkey 2)
Mut.R3 (Monkey 1)
Mut.R3 (Monkey 2)

FIGURE 8 VARIABLE REGION OF THE LIGHT CHAIN OF IOR-T 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	ASP	**ILE**	VAL	**MET**	THR	GLN	ASP	GLN	LYS	PHE	MET	SER
B	GLU	**ILE**	VAL	**MET**	THR	GLN	SER	PRO	ALA	THR	LEU	SER
C	ASP	**ILE**	VAL	**MET**	THR	GLN	ASP	GLN	LYS	PHE	MET	SER
	13	14	15	16	17	18	19	20	21	22	23	24
A	THR	SER	VAL	GLY	ASP	ARG	VAL	SER	VAL	THR	CYS	**LYS**
B	VAL	SER	PRO	GLY	GLU	ARG	ALA	THR	LEU	SER	CYS	
C	THR	SER	VAL	GLY	ASP	ARG	VAL	SER	VAL	THR	CYS	**LYS**
	25	26	27	28	29	30	31	32	33	34	35	36
A	**ALA**	**SER**	**GLN**	**ASN**	**ALA**	**GLY**	**THR**	**ASN**	**VAL**	**ALA**	**TRP**	**TYR**
B											**TRP**	**TYR**
C	**ALA**	**SER**	**GLN**	**ASN**	**ALA**	**GLY**	**THR**	**ASN**	**VAL**	**ALA**	**TRP**	**TYR**
	37	38	39	40	41	42	43	44	45	46	47	48
A	GLN	GLN	LYS	PRO	GLY	GLN	SER	PRO	LYS	**ALA**	**LEU**	**ILE**
B	GLN	GLN	LYS	PRO	GLY	GLN	PRO	PRO	ARG	**LEU**	**LEU**	**ILE**
C	GLN	GLN	LYS	PRO	GLY	GLN	SER	PRO	LYS	**ALA**	**LEU**	**ILE**
	49	50	51	52	53	54	55	56	57	58	59	60
A	**TYR**	**SER**	**ALA**	**SER**	**SER**	**ARG**	**ASN**	**SER**	GLY	VAL	PRO	ASP
B	**TYR**								GLY	ILE	PRO	ALA
C	**TYR**	**SER**	**ALA**	**SER**	**SER**	**ARG**	**ASN**	**SER**	GLY	VAL	PRO	$\boxed{\text{ALA}}$
	61	62	63	64	65	66	67	68	69	70	71	72
A	ARG	PHE	THR	**GLY**	SER	**GLY**	SER	**GLY**	**THR**	ASP	**PHE**	THR
B	ARG	PHE	SER	**GLY**	SER	**GLY**	SER	**GLY**	**THR**	GLU	**PHE**	THR
C	ARG	PHE	$\boxed{\text{SER}}$	**GLY**	SER	**GLY**	SER	**GLY**	**THR**	ASP	**PHE**	THR
	73	74	75	76	77	78	79	80	81	82	83	84
A	LEU	THR	ILE	SER	ASN	VAL	GLN	SER	GLU	ASP	LEU	ALA
B	LEU	THR	ILE	SER	ARG	LEU	GLN	SER	GLU	ASP	PHE	ALA
C	LEU	THR	ILE	SER	ASN	VAL	GLN	SER	GLU	ASP	$\boxed{\text{PHE}}$	ALA
	85	86	87	88	89	90	91	92	93	94	95	95A
A	GLU	TYR	PHE	CYS	**GLN**	**GLN**	**TYR**	**ASN**	**SER**	**TYR**	**PRO**	**LEU**
B	VAL	TYR	TYR	CYS								
C	$\boxed{\text{VAL}}$	TYR	$\boxed{\text{TYR}}$	CYS	**GLN**	**GLN**	**TYR**	**ASN**	**SER**	**TYR**	**PRO**	**LEU**
	96	97	98	99	100	101	102	103	104	105	106	107
A	**VAL**	**THR**	**PHE**	GLY	ALA	GLY	THR	LYS	LEU	GLU	LEU	LYS
B			**PHE**	GLY	GLN	GLY	THR	ARG	VAL	GLU	ILE	LYS
C	**VAL**	**THR**	**PHE**	GLY	ALA	GLY	THR	LYS	LEU	GLU	LEU	LYS
	108	109										
A	ARG	ALA										
B	ARG	GLU										
C	ARG	ALA										

FIGURE 10: VARIABLE REGION OF THE LIGHT OF IOR-CEA 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ASP	ILE	GLN	MET	THR	GLN	SER	PRO	LYS	PHE	SER	SER
B	ASP	ILE	GLN	MET	THR	GLN	SER	PRO	SER	THR	LEU	SER
C	ASP	ILE	GLN	MET	THR	GLN	SER	PRO	SER	THR	LEU	SER
	13	14	15	16	17	18	19	20	21	22	23	24
A	THR	SER	VAL	GLY	ASP	ARG	VAL	SER	VAL	THR	CYS	LYS
B	ALA	SER	VAL	GLY	ASP	SER	ILE	THE	ILE	THR	CYS	
C	ALA	SER	VAL	GLY	ASP	ARG	VAL	SER	VAL	THR	CYS	LYS
	25	26	27	28	29	30	31	32	33	34	35	36
A	ALA	SER	GLN	ASN	ALA	GLY	ILE	ASN	VAL	ALA	TRP	TYR
B											TRP	PHE
C	ALA	SER	GLN	ASN	ALA	GLY	ILE	ASN	VAL	ALA	TRP	TYR
	37	38	39	40	41	42	43	44	45	46	47	48
A	GLN	GLN	LYS	PRO	GLY	GLN	SER	PRO	LYS	ALA	LEU	ILE
B	GLN	GLN	LYS	PRO	GLY	LYS	ALA	PRO	ASN	VAL	LEU	ILE
C	GLN	GLN	LYS	PRO	GLY	GLN	SER	PRO	LYS	ALA	LEU	ILE
	49	50	51	52	53	54	55	56	57	58	59	60
A	TYR	SER	ALA	SER	SER	ARG	ASN	SER	GLY	VAL	PRO	ASP
B	TYR								GLY	ILE	PRO	SER
C	TYR	SER	ALA	SER	SER	ARG	ASN	SER	GLY	ILE	PRO	SER
	61	62	63	64	65	66	67	68	69	70	71	72
A	ARG	PHE	THR	GLY	SER	GLY	SER	GLY	THR	ASP	PHE	THR
B	ARG	PHE	SER	GLY	SER	GLY	SER	GLY	THR	GLU	PHE	THR
C	ARG	PHE	SER	GLY	SER	GLY	SER	GLY	THR	GLU	PHE	THR
	73	74	75	76	77	78	79	80	81	82	83	84
A	LEU	THR	ILE	SER	ASN	VAL	GLN	SER	GLN	ASP	LEU	ALA
B	LEU	THR	VAL	ILE	ASN	LEU	GLN	SER	ASP	ASP	PHE	ALA
C	LEU	THR	VAL	ILE	ASN	LEU	GLN	SER	ASP	ASP	PHE	ALA
	85	86	87	88	89	90	91	92	93	94	95	95A
A	GLU	TYR	PHE	CYS	GLN	GLN	TYR	ASN	SER	TYR	PRO	LEU
B	THR	TYR	TYR	CYS								
C	THR	TYR	TYR	CYS	GLN	GLN	TYR	ASN	SER	TYR	PRO	LEU
	96	97	98	99	100	101	102	103	104	105	106	107
A	VAL	THR	PHE	GLY	ALA	GLY	THR	LYS	LEU	GLN	LEU	LYS
B			PHE	GLY	GLN	GLY	THR	LYS	VAL	LEU	ILE	LYS
C	VAL	THR	PHE	GLY	GLN	GLY	THR	LYS	LEU	GLN	LEU	LYS
	108	109										
A	ARG	THR										
B	ARG	THR										
C	ARG	THR										

Table 2

```
  MOUSE HEAVY CHAINS FAMILY II
   NAME       5    10   15   20   25   30   35     40   45   50      55   60   65   70   75   80     85   90   95   100         101
 1-LB8'CL          ******                   *-******    ******                                                 ***-------------******
 2-DF4-23.4'C    **   ****                   *-******    ******                                                                ***  ***
 3-mAb D'CL           ******                   *-******    ******    *****                                                         ****
 4-BAT123'CL          ******                   *-******    ******                                                 *****--------------****
 5-35-20              ******                   *-******    ******    ***                                                              ***
 6-AN02               ********                 *-******    ******
 7-AN02'CL            ********                 *-******    ******
 8-40-120             ******                   *-******    *******---*****                                        ***------------*******
 9-40-40              ******                   *-******    *******---*****                                        ***------------*******
10-H146-24E9'    ** ******                   *-***       ******                                                                  *****
11-AN07'CL       ** ******                   *-******     *****
12-40-160            ******           *********-******    *** ***---***                                          ***------------*******
13-S1.2'CL           ******                   *-******    *******---*****                                       **--------------*****
14-40-140            ******                   *-***       *******---*****                                        ***------------*******
15-37.1.1'CL         ******                *****-******    ***                                                       ***------------*****
16-GAM3-2'CL        *******                   *-***       ******                                             ********------------***
17-8-1-12-5B'        ******                   *-******    ******                                 ******-------------------------*******
18-S27'CL            ******                   *-******    ******                                                **--------------*****
19-AN03'CL           ******                ****-******    ******                                                     **----------******
20-L11-1A1'CL        ******                   *-******    ********--******
21-AN01'CL           ******                ****-******    ******                                                       *---------******
22-VHTF'CL           ******                   *-***       ******      ***                                            **----------*****
23-MOPC 315'C        ******               *****-******    ******    *****                                                      *******
24-MOPC315           ******               *****-******    ******                                     ***                         *****
```

Table 4

```
MOUSE HEAVY CHAINS FAMILY IV
 NAME          5    10   15   20   25   30   35    40   45   50     55   60   65   70   75   80     85   90   95  100         101
1-FAC1'CL                                                ***                                                                  **  ***
2-D23'CL                                                 ***                                                                   ******
3-A8.1'CL                                                ***                                                   ****------------*******
4-A8.2'CL                                                ***                                              ***     ****--------*****
5-JV10'CL                ***                             ***                                                                 ********
6-MA-15C5'CL                                       ****  ***                                                 *****----------*****
7-163.69'CL                                              ***                                                      **--------*******
8-Mik-B1(Fv)                                       ****  ***                                                      ***--------**
9-VH101'CL                                          ***  ***                                                      ***--------***
10-MC101'CL                                         ***  ***                                                      ***--------***
11-BPPI-6.4'C   ***                                      ***
12-36.1.2D'CL                                            *** ****                                                              ******
13-36.5.7Bu'C                                            *** ****                                                              ******
```

Table 6

```
  MOUSE HEAVY CHAINS FAMILY VI
  NAME        5    10   15   20  25   30   35      40   45   50      55   60   65   70   75   80      85  90   95    100         101
 1-202.135'CL        ****               ***           **********--******                    *** ***                  *------*******
 2-202.61'CL         ****               ***           **********--******                    *** ***                **--------******
 3-202.80'CL         ****               ***           **********--******                    *** ***                          *******
 4-202s.38'CL        ****               ***           **********--******                    *** ***                          *** ***
 5-111.34'CL         ****               ***           **********--******                    *** ***                          **  ***
 6-111.109'CL    *******                ***           **********--******                    *** ***            **** *----------***
 7-17p.101'CL    *******                ***           **********--******                    *** ***         ****-------------**  ***
 8-C'CL              ****               ***           **********--******                    *** ***         *******
 9-AN11'CL           ****               ***           **********--******                                  *******                 ***
10-D44'CL            ****         **** ****           **********--******                    *** ***                              ***
11-BNA 031 VH        ****               ***           **********--********                  *** ***                 ***
12-AM29'CL           ****                **           ********                              *** ***             ******-------**  ***
13-AM28'CL           ****               ***           ******** *--*******                   ***                 ******-------**  ***
14-BWR4.H'CL                                          ********                              *** ***             **------------******
15-D444'CL           ****               ***            *********--******                   ********          **-------------******
16-PL2-8'CL          ****              ****           *****                                                     *** ***--------*****
17-PL2-3'CL          ****                             *****                                    ***              *** ***--------*****
18-PL2-6'CL          ****                             *****                                    ***              *** ***--------*****
19-34-2B'CL          ****               ***           **********--**                     ******
```

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European Patent Office
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(51) Int. Cl.^{6}: **C12N 15/13**, C07K 16/46, G01N 33/68, A61K 39/395

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **30.06.1994 CU 8094**

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(54) **Method for obtaining modified immunoglobulins with reduced immunogenicity of murine antibody variable domains, compositions containing them**

(57) Modified chimaeric antibodies, and antibody heavy and light chains, which comprise variable domains derived from a first mammalian species, usually mouse, and constant domains from a second mammalian species, usually human. Modification concerns the variable domains, in particular the framework regions of the variable domains. The modifications are made only in T-cell antigenic structures present in framework regions, and do not cover canonical structures or Vernier zone. The modifications adapt the amino acid sequences concerned to those occurring in corresponding antibodies derived from said second mammalian species. Thus, the modified chimaeric antibodies retain the original antigen recognition and binding properties but become less immunogenic to said second mammalian species, which improves their therapeutical utility with said second mammalian species. Recombinant DNA technology may be used to construct and produce the modified chimaeric antibodies.

FIGURE 1: DEDUCED AMINO ACID SEQUENCES

A VK OF MURINE R3 ANTIBODY

D V L M T Q T P L S L P V S L G D Q A S I S C **R S S Q**
N I H I V H S N G N T Y L Q W Y L Q K P G Q S P K L L
I Y **K V S N R F S** G V P D R F S G S G S G T D F T L K
I S R V E A E D L G V Y Y C **F Q Y S H V P W T** F G G G
T K L E I K R A

B VH OF MURINE R3 ANTIBODY

Q V Q L Q Q P G A E L V K P G A S V K L S C K A S G Y
T F T **N Y Y I Y** W V K Q R P G Q G L E W I G **G I N P T**
S G G S N F N E K F K T K A T L T V D E S S S T A Y M
Q L S S L T S E D S A V Y Y C T R **Q G L W F D S D G R**
G F D F W G Q G T T L T V S S

EP 0 699 755 A3

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 95 20 1752

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	EP-A-0 592 106 (IMMUNOGEN INC.) 13 April 1994 * page 7, line 10 - page 9, line 27 * * example 1 * * figure 2 * * claims * ---	1-10, 26-28	
X	PROTEIN ENGINEERING, vol.7, no.6, 4, OXFORD, GB pages 805 - 814 G. STUDNICKA ET AL. 'Human-engineered monoclonal antibodies retain full specific binding activity by preserving non-CDR complementarity-modulating residues.' * the whole document * ---	1-10, 26-28	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
A	PROTEIN ENGINEERING, vol.4, no.7, 1, OXFORD, GB pages 773 - 783 C. KETTLEBOROUGH ET AL. 'Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation.' * abstract * ---	1-15, 24-28	
A	JOURNAL OF MOLECULAR BIOLOGY, vol.235, no.1, 7 January 1994, LONDON, GB pages 53 - 60 A. CORTI ET AL. 'Idiotope determining regions of a mouse monoclonal antibody and its humanized versions.' * the whole document * ---	1-10, 26-28	
A	EP-A-0 586 002 (CENTRO DE IMMUNOLOGIA MOLECULAR) 9 March 1994 * claims * -----	12-15	

EPO FORM 1503 03.82 (P04C10)

European Patent Office

EP 95 20 1752 -B-

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims 1-9 and 12-28:

   Method for obtaining modified immunoglobulins with reduced immunogenicity of murine antibody variable domains, compositions containing them

2. Claim 10:

   Composition comprising a modified immunoglobulin having a specificity for a known antigen

3. Claim 11:

   DNA sequence encoding murine IOR-R3 antibody which recognizes EGF-R